Ultraviolet Absorption by Dipicolinic Acid in Model Systems and Bacterial Spores

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Alterations of the ultraviolet absorption spectrum of free dipicolinic acid can be effected by varying the dielectric constant of the medium in which it is dissolved. These alterations simulate the spectrum obtained upon calcium chelation. Suppression of the ionization of the carboxyl groups on the pyridine ring by esterification also influences the spectrum of the acid. The modified spectra, in some instances, show absorption maxima in the same regions as those observed by studying the absorption of intact bacterial spores in water.

A fundamental problem in bacterial spore research concerns the determination of the contribution of 2,6-pyridinedicarboxylic acid, commonly known as dipicolinic acid (DPA), to spore structure. It is well established that DPA is essential to the heat resistance and dormancy of the spore form (1), but the chemistry involved in the establishment of these properties is unknown.

Studies of exudates of germinating spores (2), heated spores (3), mechanically disrupted spores (4), and chemically treated spores (3) have resulted only in the isolation of DPA in the form of its calcium salt. All this, plus recent study of the ultraviolet absorption spectra of intact spores embedded in KBr (5), has led to assumptions that the DPA in intact spores may exist there primarily as calcium dipicolinate.

Even though the ultraviolet absorption spectrum of intact spores has been found similar to the well-studied spectrum of calcium dipicolinate (6), little is recorded as to other factors that could possibly alter the absorption spectrum of free DPA in such fashion that it would appear similar to that of the calcium salt.

Our paper presents data describing alterations of the absorption spectrum of free DPA effected by changes in the dielectric constant of the medium in which it is dissolved. The spectrum of DPA dissolved in alcoholic solutions having low dielectric constants with respect to water is compared with the spectrum of calcium dipicolinate, the diethyl ester of DPA and intact spores suspended in water.

MATERIALS AND METHODS[1]

The four alcohols used in our study were methanol, ethanol, 1-propanol, and 2-propanol. They were of the "highest purity" commercial grade and were not redistilled and dried prior to use.

DPA was obtained from the Aldrich Chemical Company, Inc., Milwaukee, Wisconsin, and was used without further purification.

The following spores were used: *Bacillus subtilis* 15u, American Can Co.; *Bacillus cereus* NRS 804, and *Bacillus megaterium*, QMB 1551. *B. subtilis* was produced according to the method described by Harper *et al.* (7). *B. cereus* and *B. megaterium* were produced in G medium as described by Hasimoto *et al.* (8). Spore crops were washed a minimum of eight times with demineralized water at 2-4°, lyophilized, and stored in a desiccator prior to use.

A series of sample solutions of increasing alcohol concentration by weight were prepared containing a constant amount (40 μg/ml) of DPA.

[1] Reference to certain products or companies does not imply an endorsement by the Department over others not mentioned.

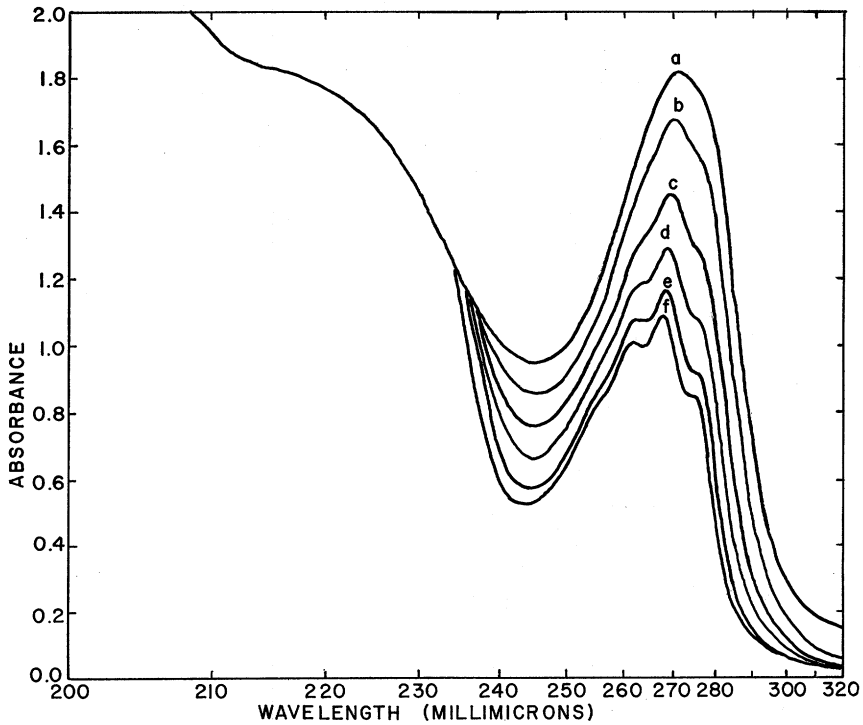

FIG. 1. Effect of increasing amounts of ethanol (v/v) on the absorption spectrum of an aqueous DPA solution. DPA concentration: 40 μg/ml. a, 10% ethanol; b, 30% ethanol; c, 50% ethanol; d, 70% ethanol; e, 90% ethanol; and f, absolute ethanol.

The ultraviolet absorption of these samples was measured by use of a Perkin-Elmer Model 350 Double Beam Spectrophotometer, equipped with a quartz cell having a 1-cm light path. An identical series of solutions containing no DPA served as blanks. All measurements were made at room temperature (25°).

The mono- and diethyl-esters of DPA were prepared by dissolving pure DPA in absolute ethanol and catalyzing the reaction with dry gaseous HCl as described by Barnes and Fales (9). The esters were then separated and purified by distillation and subsequent recrystallization.

In order to prepare spore suspensions free of DPA, 10-mg samples were dispersed in 5 ml demineralized water, autoclaved 15 minutes at 15 psi, thoroughly washed in demineralized ice water, and resuspended in 10 ml ice water. Similar suspensions of intact spores were subjected to the same washing procedure. Ultraviolet absorption spectra of suitable dilutions of these suspensions were then obtained, using the DPA-free suspension in the reference chamber of the spectrophotometer.

RESULTS

Figure 1 shows the effect of ethanol on the absorption spectrum of an aqueous DPA solution. As the concentration of ethanol increased, two distinct shoulders appeared at 275 and 262 mμ, with a large peak remaining at 268–270 mμ. Curves similar to these were obtained with all alcohols investigated. A scan of an aqueous calcium dipicolinate solution yielded absorption maxima at wavelengths identical with those obtained with alcoholic DPA solutions. The addition of ethanol had no effect on the calcium dipicolinate absorption curve.

Figure 2 is a plot of the ratio of the peak heights at 270/275 versus the dielectric constants at 25° for solutions of four alcohols containing the same amount of DPA. The dielectric constants were calculated from the data of Akerlof (10) for aqueous solutions of the alcohols before the addition of DPA. It can be seen that the solutions with a dielectric constant above 50 (corresponding to an alcohol concentration of 50 weight % and below) have approximately the same peak ratio. At higher concentrations of alcohol, there is less agreement, probably due to

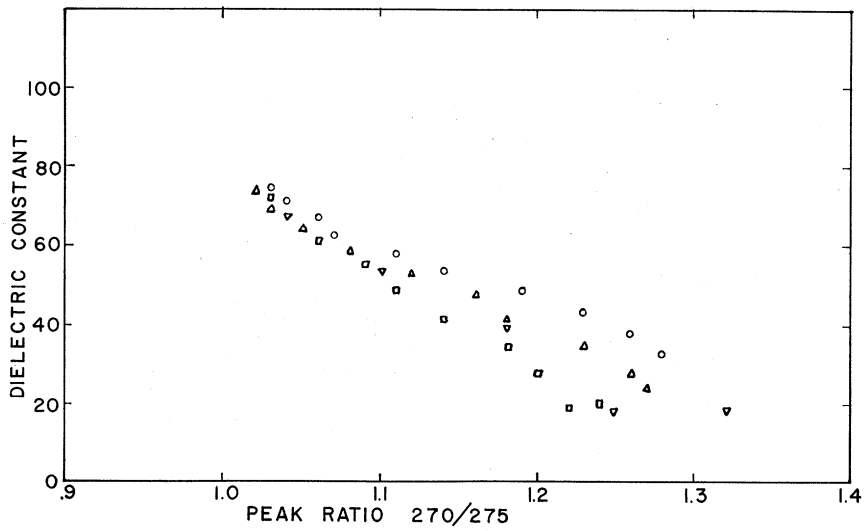

FIG. 2. Peak height ratio versus dielectric constant of solutions of four different alcohols at 25°. ○, Methanol; △, ethanol; □, 1-propanol; ▽, 2-propanol.

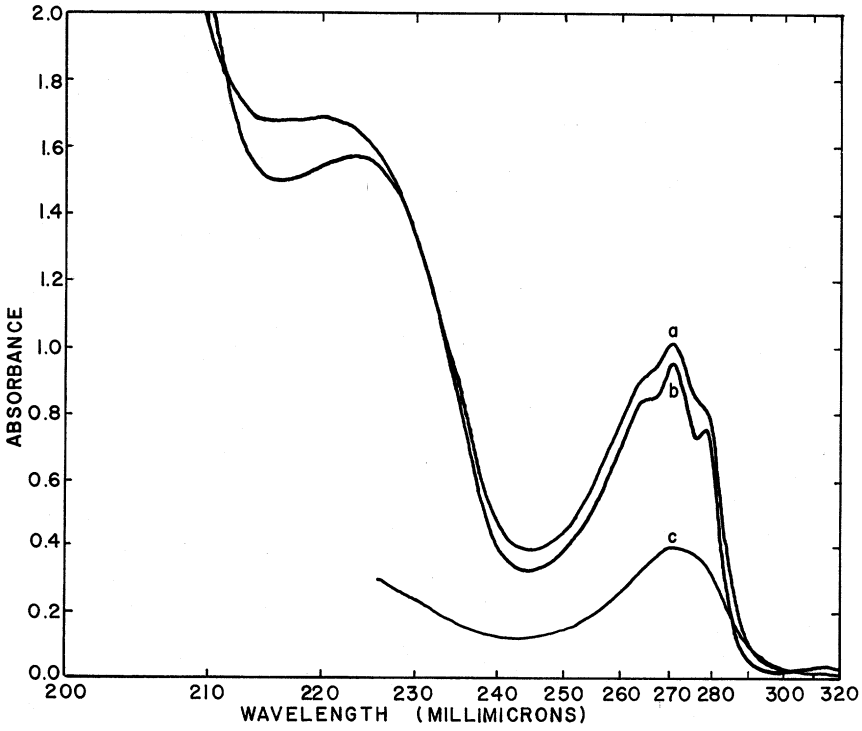

FIG. 3. Absorption spectra of the mono- and diethyl esters (conc., 40 μg/ml) of DPA compared with the spectrum of pure DPA (conc., 10 μg/ml). a, Mono-ethyl ester; b, diethyl-ester; c, DPA.

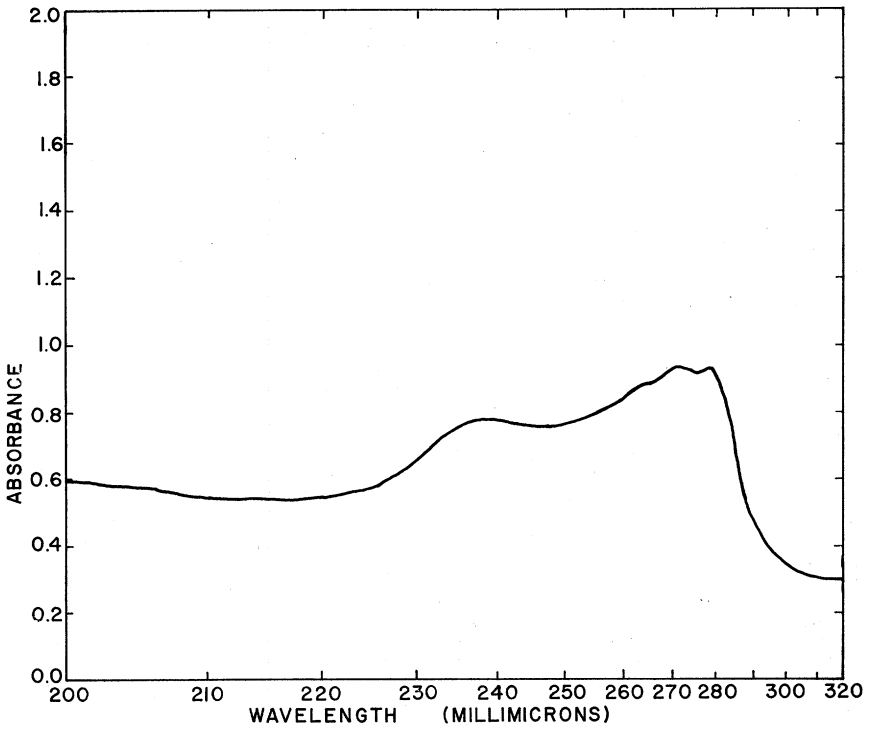

FIG. 4. Absorption spectrum of an aqueous suspension of intact *B. subtilis* (conc., < .75 mg spores/ml).

factors other than the effect of decreasing the dielectric constant.

Figure 3 shows the absorption spectra of aqueous solutions of the mono- and diethyl-esters of DPA. It can be seen that the monoethyl ester (m.p. 123–125°) exhibits a maximum at 272 mμ with distinct shoulders at 266 and 278 mμ, which agree with those previously published by Perry and Foster (11). The diethyl ester (m.p. 41–42°) exhibits absorption maxima at 278 and 270 mμ with a shoulder at 264 mμ. It is obvious that the addition of the second ester linkage causes a marked increase in the absorbance at 278 mμ. Samples were scanned immediately after dissolution to eliminate any possibility of hydrolysis of the ester linkages on standing. Examination of ethanolic solutions of the two esters yielded identical absorption curves to those obtained in aqueous solution. The spectrum of an aqueous solution of pure DPA is shown for comparison.

Figure 4 shows the result of a double-beam scan of an aqueous suspension of intact spores (*B. subtilis*) against a reference suspension containing an equal weight of DPA-free spores. A broad absorption band may be observed with maxima at 279 and 271 mμ and a shoulder at 262 mμ. Similar traces were obtained with *B. cereus* and *B. megaterium*. The same samples yielded spectra with maxima at 280 and 271 mμ and a shoulder at at 262 mμ upon suspension in absolute ethanol.

Examination of the aqueous supernatant of the intact spores indicated no DPA leakage during the experiment. No leakage occurred after holding an aqueous suspension of washed intact spores overnight in a refrigerator.

DISCUSSION

From the results obtained in this study, it can be seen that changes in the ultraviolet absorption spectra of DPA, similar to those observed on calcium chelation, can be obtained by mechanisms which tend to suppress the ionization of the carboxyl groups on the pyridine ring.

Yasuda (12) has demonstrated that suppression of carboxyl group ionization can be observed on decreasing the dielectric constant of the acidic solutions by addition of alcohols. His work also shows that an acid has nearly the same dissociation constant in various media having the same dielectric constant.

The proposed direct relationship between the extent of ionization of the carboxyl groups of DPA, as governed by the dielectric constant of the solvating medium and the development of fine structure in the DPA spectrum, holds well in all solutions containing 50% alcohol or less, as shown in Fig. 2. The differences in spectra observed in solutions containing more than 50% alcohol may result from the differences in the ability of the various alcohols to change the solvation shell of the DPA molecule.

A brief study of the pH of methanolic solutions of DPA was made to assure ourselves that decreasing dielectric constant of the medium suppressed ionization of this acid, as observed with other acids by Yasuda (12). In a solution containing a fixed concentration of DPA, the pH increased linearly as the methanol content was increased. The observations were stopped at solutions containing 80% methanol since serious errors are introduced in pH measurements made with a glass electrode above this alcohol concentration (13).

Since esterification of the carboxyl groups also develops fine structure in the absorption spectrum of DPA, as shown in Fig. 3, the possibility existed that the spectral shifts we observed in alcoholic solutions resulted from partial or complete esterification. Although this was highly unlikely, the possibility was ruled out by determining the absorption spectra of DPA in solutions of methyl acetate, ethyl acetate, and methyl formate. These spectra were identical to those obtained in alcoholic solutions.

The problems encountered in the determination of absorption in translucent materials are well-documented, and a number of solutions have been proposed (14–17). The principal problems arise from attenuation of the transmitted beam by scattering and preparing suitable blanking systems.

In their study of the absorption of ultraviolet light by intact spores, Bailey *et al.* (5) overcame much of the problem induced by scattering by suspending the spores in KBr, which has a refractive index near that of the bacterial spores. To blank out the absorption by spore components other than DPA, KBr discs containing the coats of disrupted spores were used in the reference beam of the spectrophotometer. Since this technique did not blank out the considerable absorption by nucleic acids in the spore, we tried another approach.

When the spores are heated in water, all DPA is released without loss of any other major ultraviolet-absorbing constituents (18). When suspensions of these spores were used in the reference beam of a spectrophotometer to both blank out ultraviolet absorption by spore components other than DPA and balance the attenuation due to scattering, the spectrum shown in Fig. 4 was obtained. The absorption peaks are broad and ill-defined with maxima in the regions observed in alcoholic solutions of DPA. Whether this spectrum truly represents that of the difference in retractility of heated and unheated spores continues under investigation.

Even though it can now be seen that certain ambiguities could arise in interpretation of the spectrum of DPA located in poorly defined systems, studying the ultraviolet absorption of intact bacterial spores is of interest. It is apparent, however, that spectral absorption data obtained from investigations of intact spores must be carefully considered before they can be used to define the bonds effective in retaining DPA in the spore structure.

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